

T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (*Holothuria scabra*): Possible involvement in differential recognition of bacteria

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Abbreviations

HSL, *Holothuria scabra* lectin; Me α Gal, methyl- α -D-galactopyranoside; T-antigen (Gal β 1-3 GalNAc α -1-O-L-Ser), Thomsen-Friedenreich antigen.

Abstract

In invertebrates, cellular and humoral components are evolved to maintain their body immunity and integrity. Both these factors respond to different antigens such as microorganisms, vertebrate erythrocytes and foreign proteins. In this article we report a study of a lectin (HSL) involved in immune response in the echinoderm, sea cucumber (*Holothuria scabra*). Correlative studies indicate that the expression of this defensive lectin is induced by bacterial challenge, wherein cell wall glycoconjugates of bacteria are involved in lectin induction. HSL showed strong broad spectrum antibacterial activity against both gram-positive and gram-negative bacteria. Under in vitro conditions, purified HSL mediate agglutination of the test bacteria, there by indicating a possible mode of action in physiological situation.

Keywords: Sea cucumber; *Holothuria scabra*; coelomic fluid; lectin; agglutination; antibacterial activity.

1. Introduction

Marine invertebrates rely solely on innate immunity, which includes both humoral and cellular responses, as they lack an adaptive immune system. Various methods employed to counteract infectious agents include, hemolymph coagulation, melanization, cell agglutination, encapsulation, nodule formation and phagocytosis (Cooper et al., 1992). The microbial load in natural marine habitat can number up to 10^6 bacteria and 10^9 virus mL^{-1} of seawater (Ammerman et al., 1984). It is therefore imperative that animals develop a robust innate immune system for survival.

Lectins are proteins with specificity for simple sugar, a sequence of sugars or their glycosidic linkages (Bretting & Kabat 1976). Such ligands are known to occur on the surface and the capsule of bacteria. In invertebrates lectins have been suggested to participate in innate immune response (humoral defense reaction) by inducing bacterial agglutination or by acting as opsonins to enhance phagocytosis by coelomocytes (Bayne 1990). Besides role in cell recognition and host defense, lectins have long been used as probes to determine sugar composition of glycan and glycoconjugates like bacterial lipopolysaccharide, cell surface glycoproteins and glycolipids (Gabijs et al., 1998). Cell lysates and cell-free plasma of several invertebrates also expressed antibacterial activity, although the activity of the latter may possibly be due to small antimicrobial proteins (Stabili et al., 1996). These results strongly support the contention that invertebrates possess “immune-like” defense mechanisms.

Sea cucumbers are echinoderms inhabiting relatively shallow coastal areas experiencing little or no currents. The coelomic fluid of the holothuroid is known to contain naturally occurring factors exerting hemagglutinating and hemolytic activity

against various erythrocytes and microorganisms (Parrinello et al., 1976). The nature of interaction of lectins with antigens such as, bacterial cells, and their significance in innate immunity has not been elucidated in detail, although many lectins for bacteria have been detected in a variety of invertebrates (Cassels et al., 1986; Olafsen et al., 1992; Zipris et al., 1986). Earlier we have isolated, purified and characterized with respect to its biochemical and carbohydrate-binding properties of a lectin from coelomic fluid of sea cucumber (*Holothuria scabra*) upon immune challenging. *H. scabra* lectin (HSL) is a monomeric glycoprotein weighing 182 kDa, which does not require any divalent cations for its hemagglutinating activity. It agglutinates pronase treated human erythrocytes of all human blood groups. It is highly stable at high temperature and under wider pH range. HSL is T-antigen specific, can distinguish α - from β -anomers (Gowda et al., 2008). In this article, we report our studies on induction of HSL upon immune challenging and its interaction with both gram negative and gram positive human pathogenic bacteria and demonstrate the involvement in innate immunity.

2. Materials and methods

2.1. Materials

Methyl α -D-galactose (Me α Gal), phenyl sepharose, trypsin, ampicillin, phenylmethylsulphonylfluoride (PMSF) were from (Sigma Chemical Co. St. Louis, U.S.A); all other chemicals used were of analytical grade.

2.2. Immune challenge and preparation of coelomic fluid

Adult sea cucumbers (*H. scabra*) were collected from Anjuna coast of Goa, India and maintained in circulating seawater for at least 3-4 days. During exposure animals

were kept in fiber-plastic tanks containing 50 L seawater; with two change of water everyday, care was taken to ensure that the animals were not much disturbed during the experiment. Except for the treatment under consideration, all other general environmental conditions were similar (salinity of seawater, 35 ± 5 PPT and temperature 28 ± 2 °C).

Bacteria used in this experiment are human isolates *Staphylococcus citreus* and Group D *Streptococci* (gram positive), *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marganii*, *Pseudomonas aeruginosa* and *Proteus vulgaris* (gram negative) obtained from Goa Medical College, Goa, India. Marine isolates *Vibrio cholerae*, *Shigella* sp. (gram negative) and *Bacillus* sp (gram positive), were isolated in the lab. All bacterial cultures were grown in nutrient broth, cells were harvested separately by centrifugation at 6,000 g for 8 min, washed thrice, and resuspended in brine saline (0.85 % NaCl, w/v). Bacterial count was estimated by serial dilution method, to know the exact amount of bacteria used for challenging. About 10^{14} CFUs of each isolate was separately injected into the coelomic cavity of 24 animals. On each day 3 animals are sacrificed and coelomic fluid was collected from each challenged animal separately at regular time intervals (1, 2, 3, 4, 5, 6, 7 and 8th day), in 1:1 ratio with Alseiver's solution as anticoagulant (dextrose 20.5 gm, tri sodium citrate 8 gm, citric acid 0.55 gm and sodium chloride 4.2 gm in 1000 mL water). Control included a set of animals injected with brine saline only. The resulting coelomic fluid was centrifuged (5000 g, 15 min at 4 °C) to remove coelomycetes and the clear supernatant was treated with serine proteinase inhibitor (PMSF, $20 \mu\text{g}\cdot\text{mL}^{-1}$) and stored at -20 °C. Coelomic fluid from unchallenged animals served as control. After the 8th day, the same sets of animals were rechallenged as before to determine the presence of any immune memory.

2.3. Estimation of residual bacterial load

Prior to processing, an aliquot was removed from the coelomic fluid preparation to estimate the residual bacterial load by serial dilution method. This was used as an index for in vivo anti-bacterial activity.

2.4. Hemagglutination assay (HA)

Human erythrocytes of blood group A, B and O were washed 4-5 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl (TBS) and a final suspension of 3 % (v/v) was prepared in TBS and treated with pronase (0.5 mg mL^{-1}), incubated at 37°C for one hour and later washed 4 times with TBS before use.

Hemagglutination tests were performed in standard microtitre plates with U-bottom wells by two-fold serial dilution method. A 50 μl aliquot of erythrocyte suspension was mixed with 50 μl of serially diluted coelomic fluid, incubated for 1 h and visually examined for agglutination. The unit of activity (HA units) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination.

2.5. Purification of the lectin

The HSL, T-antigen specific lectin was purified to homogeneity by two-consecutive hydrophobic interaction (phenyl sepharose) column chromatography (Gowda, N. M. et al. 2008). Briefly, the coelomic fluid was collected from bacterial challenged sea cucumber animals on dissection, and processed immediately to separate coelom from coelomycetes. Coelomic fluid preparation was concentrated over a 10 kDa ultra filtration membrane (to remove $<10 \text{ kDa}$ contaminants) and heat treated at 60°C for 2 h to inactivate proteases. Tris-HCl buffer, pH 8.5 (TB) was added to a final volume

concentration of 20 mM. Solid ammonium sulphate was added to the preparation to a final concentration of 3 % (w/v). The sample was then passed through phenyl sepharose column, pre-equilibrated with TB containing 3 % (w/v) ammonium sulphate. The column was washed with loading buffer until A_{280} of elute returned to baseline level. The bound lectin was eluted with TB. Fractions showing hemagglutination activity were pooled together, ammonium sulphate added to a final concentration of 1 % (w/v) and loaded on phenyl sepharose, pre-equilibrated with TB containing 1 % (w/v) ammonium sulphate. Bound protein was eluted with TB. Fractions with lectin activity were pooled together and stored at 4 °C until further use. The confirmation of the homogeneity and activity of the lectin were done.

2.6. Agglutination of bacteria

Above mentioned bacterial strains were grown in nutrient broth at 37 °C for 16 h, harvested by centrifugation at 2000 g for 10 min, washed thrice with TBS and suspended in same buffer. Bacterial count was calculated by serial dilution method and A_{620} was maintained around 1.0. Bacterial agglutination was tested by mixing 100 µl of coelomic fluid or HSL (10 µg·mL⁻¹) to an equal volume of bacterial suspension. In other set HSL was incubated with 20 mM of MeαGal as a ligand protection (A monosaccharide which specifically binds to HSL with higher affinity) for 30 min and later mixed with equal volume of bacterial suspension. Results were observed microscopically after incubating for 12 h and compared with bacterial suspensions incubated in the absence of coelomic fluid or HSL.

In order to associate agglutination with lectin activity, two-fold dilution of all the bacterial suspensions were added with 1:1, 1:2, 1:4 ratios of coelomic fluid or HSL

(lectin: bacteria), incubated for 1 h, centrifuged for 5 min at 12,000 g and the supernatant tested for residual hemagglutination activity.

2.7. Bacterial glycoconjugates agglutination test

Glycoconjugate agglutination test was performed by modification of the method described by (Kellens et al., 1993). In brief, bacterial cells were grown in three sets of 20 ml each in nutrient broth for 6 h at 37 °C. Cells were collected by centrifugation at 2000 g for 10 min, washed thrice and suspended in TBS. A_{620} of the suspensions were maintained at 1.0. The cells were used in 3 sets of experiment. A set of cells was left untreated as a control. Second set was treated with trypsin ($0.1 \text{ mg}\cdot\text{mL}^{-1}$, incubated at 37 °C for 1 h) for partial hydrolysis of proteoglycans and thereby enhancing the surface availability of glycan residues. Third set was subjected to drastic condition (cell pellets was resuspended in 250 mM glycine-HCl buffer, pH 2.0 and boiled for 15 min) so as to degrade surface structures and dissolve more cell wall glycoconjugates. The treated and untreated samples were assayed for agglutination under standard assay conditions.

2.8. Antibacterial activity

Antibacterial activity of coelomic fluid and HSL was checked under in vitro conditions by the agar adsorption technique, bacteria were cultured in nutrient broth for 24 h, and for each test the culture medium was diluted 10-fold for adjusting A_{660} to 1.0. Direct adsorption technique was performed by making wells in the agar plates and known quantity of HSL (5, 10, 25, 50 and 100 µg, positive control (ampicillin 10 µg) and a negative control) was poured on to each well, and allowed for absorption of sample by the agar. Sterile cotton bud was then dipped in a bacterium culture, and the inoculums

were spreaded uniformly on the surface of the agar media to produce a bacterial field. Each test was performed in duplicates.

Antibacterial activity was also measured by the method as previously described (Olafsen, J. A. et al. 1992). Each bacterial cell suspension was diluted to 10^4 cells mL⁻¹ with nutrient broth. Several concentrations of HSL were added into a 2 mL broth, incubated for 6 h at 37 °C. The growth of bacteria was expressed as the turbidity, as measured at A_{620} . Comparison of antibacterial activity against different bacteria was performed using the ratios of different concentrations of HSL giving 50 % inhibition of bacterial growth.

3. Results and discussion

Humoral lectins are believed to participate in immune response in invertebrates. They serve to opsonize the invading bacterium or foreign particle and thereby enhancing its phagocytosis by coelomycetes. Only few experiments have showed a precise mechanism of recognition with bacteria such as the horseshoe crab lectin, *Carcinoscorpius rotundicaudia*, recognizes the 2-keto-3-deoxyoctonate group in the cell wall polysaccharide of *E. coli* (Dorai et al., 1982). *Sambucus nigra* agglutinin can agglutinate 1, 1/2, 2, 14, 15 and 16 stereotypes of *Streptococcus suis* out of 35 strains tested due to the presence of possible Neu5Ac- α (2, 6) GalNAc sequence (Charland et al., 1995), even wheat germ agglutinin, v.gr., has agglutinated *P. haemolytica* biotype T and capsular sereotype 3, 4 and 10, due to the presence of *N*-acetylated residues or their oligomers on their cell wall determinants (Craft et al., 1987).

A number of lectins have been isolated from echinoderms that are capable of interacting with erythrocytes. However, little is known about the mechanism of

interaction with bacteria and their role in innate immunity. In our studies on sea cucumber, we observed upregulation in the expression of a humoral lectin in response to artificial challenge with bacteria. Sea cucumbers, maintained in seawater-aquaculture, were separately challenged with 10^{14} CFUs of ten different bacteria and their coelomic fluid examined over a period of eight days. In most cases the bacterial load dropped to $\sim 0.3 \times 10^6$ CFU's within one day and to negligible level by the fifth day of post-inoculation (Table 1), indicating progressive clearance of bacterial load in the coelomic cavity of the animal. However, in case of *Serratia sp.*, *E. coli* and *Bacillus Sp.* the clearance on day one was slightly lower than that of other strains but became comparable from second day onwards.

3.1. Hemagglutination activity in coelomic fluid

Hemagglutinating activity of the coelomic fluid was monitored over a period of 8 days in both challenged and unchallenged animals. Agglutination activity was determined by measuring the lowest titre producing 50 % hemagglutination. The activity is expressed as the reciprocal of the titer of each fraction. On day one, post-inoculation, the fluid from challenges with *Serratia sp.*, *Klebsiella sp.*, *Shigella*, *E. coli*, *Staphylococcus sp.* and Group D *Streptococci* showed an increased hemagglutinating activity ($\sim 2.25 \times 10^3$ HA.units mg^{-1}) as compared to rest of the challenges ($\sim 0.65 \times 10^3$ HA.units mg^{-1}). The HA progressively increased till the 5th day ($\sim 50.0 \times 10^3$ HA.units mg^{-1}), and thereafter returning to the basal level by the 8th day (Table 2). Coincidentally on the same 5th day, we had observed clearance of bacteria in the coelomic cavity of the animal. This clearly demonstrates a strong correlation between lectin expression and bacterial clearance. All the test bacteria were cleared in about the same time frame ascribing broad specificity to

the induced lectin. The protein content increased in the coelomic fluid from challenged animals as compared to that of control. These results clearly show that the hemagglutinating activity in the coelomic fluid of the challenged animals increased greatly after bacterial injection. It is possible that bacterial challenge induced higher production of coelomocytes and thereby synthesizing and releasing higher quantity of lectin into coelomic fluid. A similar pattern was observed on in vivo challenging of Pacific oyster hemolymph by *Vibrio anguillarum* resulted in increased hemolymph lectin activity (Olafsen, J. A. et al. 1992). In the horseshoe crab, bacterial lipopolysaccharide (LPS) induces exocytosis by granular hemocytes resulting in the secretion of various defensive molecules, such as lectins and antimicrobial peptides, via a G protein-mediated signaling pathway (Ozaki et al., 2005).

Immune system in vertebrates is characterized by the unique property of memory, whereby a second antigen challenge results in immediate response. In order to examine the involvement of memory in the induction of this immune lectin, we rechallenged the test animals on the 9th day; by this time, primary infection had been fully cleared and the associated increase in hemagglutination activity had subsided to basal level. However, the pattern of clearance and the corresponding increase in hemagglutination activity was as in primary challenge. This is indicative of absence of memory-based immunity in sea cucumbers. Previous studies have indicated such an absence in echinoderms (Relf et al., 1999).

3.2. *Bacterial agglutination*

Coelomic fluid and purified lectin were tested for their ability to agglutinate the test bacteria. Microscopic observation revealed that co-incubation induced agglutination

of bacterial cells. Sets of samples were subjected to trypsinization and acid-heat treatment to obtain uniform dispersion. For quantitation, the samples were centrifuged and the hemagglutination activity of the supernatant determined. Residual activity was inversely proportional to the amount of lectin adsorbed to bacterial cells. Bacterial agglutination was also observed in sialic acid binding lectin from Horse Mussel (*Modiolus modiolus*) hemolymph which agglutinated *Vibrio* spp. (Tunkijjanukij et al., 1997).

Bacterial agglutination due to coelomic fluid and HSL was observed in untreated, trypsinized and acid-heat treated cells. Untreated cells showed auto-agglutination preventing visualization of lectin-mediated agglutination partially. Trypsinization resulted in fine aggregates, which could be visualized easily. On the other hand, acid-heat treatment resulted in a range of coarse to fine aggregates. Out of the ten bacteria tested, HSL mediated agglutination was evident with untreated cells of *E. coli*, *Staphylococcus* sp., and Group D *Streptococci* (Fig.1 A, B, C). Agglutination with *Serratia* sp., *Klebsiella* sp. and *Shigella* sp., were observed when subjected either to trypsinization or acid-heat treatment. A lectin from shrimp (*Penaeus japonicas*) showed similar pattern of broad spectrum bacterial agglutination against both gram-positive and gram-negative bacteria (Yang et al., 2007). No agglutination was noticed on co-incubation of HSL with *Pseudomonas* sp., *Proteus* sp, *Bacillus* sp. and *Vibrio* sp., despite pre-treatment and non induction of lectin in coelom of the animal by these strains clearly indicates that, the lectin was not binding to any of these cell surface glycoconjugates. However, the broad spectrum scavenging of all bacteria in the coelom may be due to presence of either antibacterial peptides and/or other lectins also. Reduction in bacterial agglutination was observed when lectin was pre-incubated with 20 mM, Me α Gal (Fig 1D). The bacterial

agglutination might be due to the presence of cognate glycan antigen on the cell surface of induced bacteria. For instance, D-galactose is known to occur as a moiety in the O-antigen of *E. coli* (Vinogradov et al., 1999).

3.3. *Anti bacterial activity*

Assay of antibacterial activity with purified HSL and cell free coelomic fluid of sea cucumber, revealed that the HSL exhibited strong antibacterial activity against both gram positive and gram negative bacteria. 5 µg of HSL was able to inhibit efficiently the growth of *Staphylococcus* sp. as indicated by decrease in 50 % of turbidity, whereas 15 µg inhibited complete growth. HSL also inhibited effectively the growth of other gram negative (*Serratia* sp., *Proteus* sp., *Shigella* sp., and *E. coli*) and gram positive (Group D *Streptococci*) bacteria (Fig. 2), indicating its broad spectrum antibacterial effect. These results are in accordance with the bacterial agglutination and residual hemagglutination assays and in vivo antibacterial activity. This clearly demonstrates that the HSL preferentially binds to the glycoconjugate moiety on the cell surface of the bacteria, thereby enhancing the phagocytosis rate and /or bactericidal activity. Lectin from Horse mussel exhibited strong antibacterial effect against *Vibrio* spp. (Tunkijjanukij & Olafsen 1998). Galactose-binding lectin from *Polyandrocarpa misakiensis* showed strong antibacterial activity (Suzuki et al., 1990).

In conclusion, we show for the first time the involvement of a high molecular weight monomeric agglutinin in the innate immunity of *H. scabra*. HSL can agglutinate gram +ve and gram -ve bacteria and pronase treated human erythrocytes. It exhibited strong antibacterial activity both under in vivo and in vitro conditions. It is promising to explore the molecular aspects of phagocytic and opsonic effect of HSL in innate

immunity of sea cucumbers. We are presently working on cloning and domain architecture of this lectin.

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Figure legends

Fig.1. Agglutination of Group D *Streptococci*. Agglutination test for Group D *Streptococci* was done by incubating with equal volume of purified HSL, (A) Control cells plus 25 μ l TBS buffer; (B) early stage of agglutination after incubating with 10 μ g of HSL; (C) complete agglutination after 12 h of incubation; (D) bacteria plus 10 μ g of HSL and 25 μ l of 20 mM Me α Gal. Magnification: 40x, bar; 250 μ m.

Fig.2. Antibacterial activity of HSL against different bacteria stains. The antibacterial activity against both gram positive and gram negative bacteria was measured as the turbidity at 620 nm after incubation for 6 h with the difference concentrations of HSL. The symbols used are; *Staphylococcus* sp. (■), *Streptococcus* sp. (●), *Shigella* sp. (▲), *Klebsiella* sp. (▼), *Serratia* sp. (◄), *E. coli* (□).

Figure 1

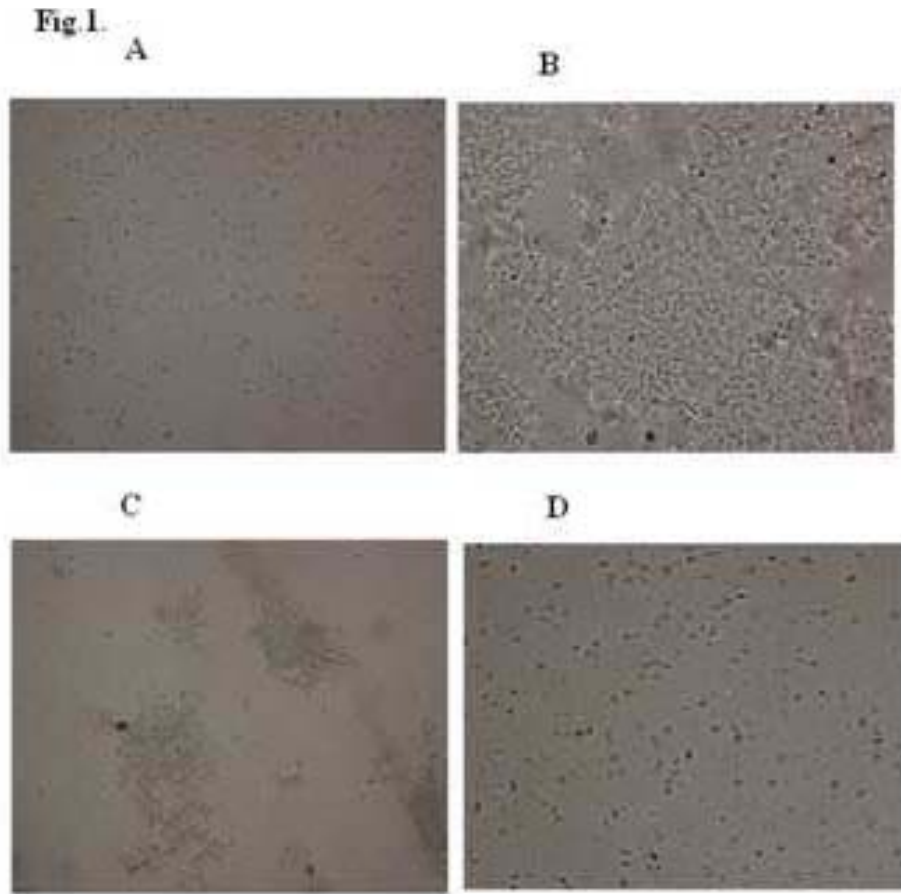


Fig.2

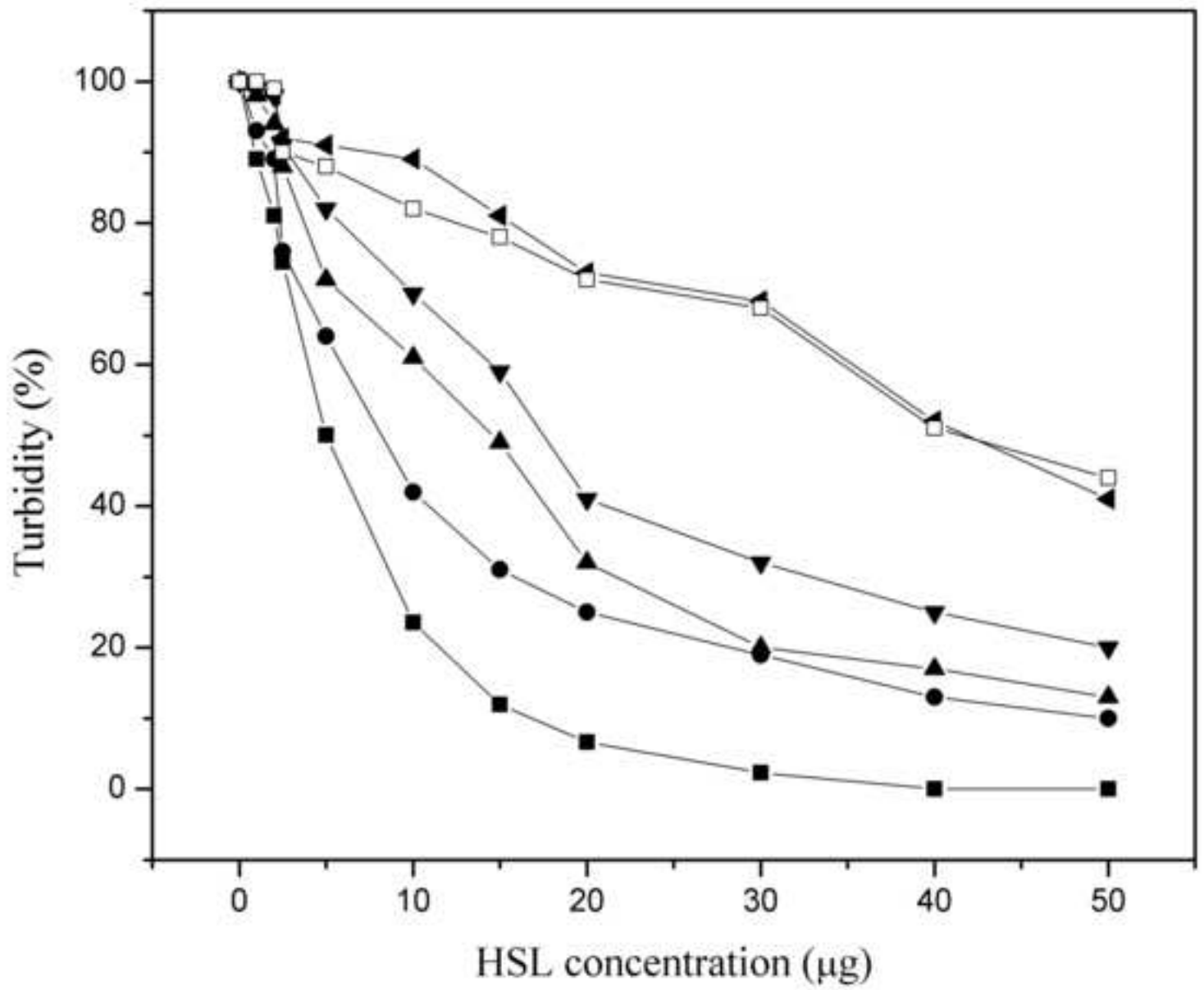


Table 1

Table 1. Residual bacterial count as function of time. The bacteria were injected into the coelomic cavity of *Holothuria scabra* maintained in aquaculture. Coelomic fluids were collected on regular interval and tests for residual bacterial count.

Bacterial strains	Injected	Day 1	2	3	4	5
	(CFU x 10 ¹⁴)	(CFU x 10 ⁶)	(CFU x 10 ⁴)	(CFU x 10 ⁴)	(CFU x 10 ³)	(CFU x 10 ³)
Control	--	0.01 x10 ⁴	0.03	0.03	0.10	0.10
(unchallenged)						
<i>Serratia</i> sp.	0.56	2.02	0.08	0.48	0.43	0.41
<i>Klebsiella</i> sp.	0.51	0.13	0.07	0.96	0.93	0.83
<i>Shigella</i> sp.	0.50	0.13	0.03	0.29	0.26	0.31
<i>E. coli</i>	0.49	0.96	0.21	0.02	0.02	0.10
<i>Vibrio</i> sp.	0.52	0.07	0.29	0.04	0.04	0.07
<i>Pseudomonas</i> sp.	0.48	0.02	0.19	0.02	0.02	0.02
<i>Streptococcus</i> sp.	0.52	0.05	0.46	0.06	0.08	0.06
<i>Proteus</i> sp.	0.51	0.07	0.24	0.96	0.83	0.61
<i>Bacillus</i> sp.	0.54	1.18	0.79	0.32	0.48	0.14
<i>Staphylococcus</i> sp.	0.49	0.11	0.05	0.01	0.02	0.09

Table 2

Table 2. Hemagglutinating activity expressed as HA.units mg⁻¹ of *H. scabra* coelomic fluid with pronase treated human erythrocytes after challenging with different bacterial strains for varying days of duration.

Bacterial strain	(10 ³ x HA units. mg ⁻¹)		
	Day 1	Day 5	Day 8
Control (unchallenged)	0.38	0.51	0.54
<i>Serratia</i> sp.	2.04	25.00	1.19
<i>Klebsiella</i> sp.	2.27	33.33	1.44
<i>Shigella</i> sp.	1.05	50.00	1.07
<i>E.coli</i>	1.05	25.00	1.20
<i>Vibrio</i> sp.	0.61	0.57	0.51
<i>Pseudomonas</i> sp.	0.68	1.09	0.51
<i>Streptococcus</i> sp.	1.32	50.00	1.88
<i>Proteus</i> sp.	0.62	0.61	0.49
<i>Bacillus</i> sp.	0.95	1.47	0.57
<i>Staphylococcus</i> sp.	1.88	100.00	1.45